INNOVATIONS

The Use of *Kryptolebias marmoratus* Eggs as an Educational Tool for Embryology Education

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Abstract: Plastic embryological models lack the excitement of seeing real, live embryos. Chick embryos are often used to demonstrate embryological development and blood circulation to students but this necessitates the death of the organism. *Kryptolebias marmoratus* embryos are large and can be viewed by means of a light microscope without need to harm the organism. Eggs are easily obtained from captive fish; and are easy to display to students. Embryological features common to early human development are readily visible in the *K. marmoratus* embryos: early brain development, eye development, somites, limb buds, blood circulation and pigment cell migration. Embryos also twitch when exposed to light and the beating heart is readily viewable. Use of the embryos in a class room has proven rewarding, students marvel at observing embryological development and are more eager to consider the common embryology and evolution of chordates.

Key Words: *Kryptolebias*, embryos, gastrula, vitelline artery, melanophore, erythrophore, somites

INTRODUCTION

Kryptolebias marmoratus (Poey, 1880) are small hermaphrodite fish found in mangrove swamps along the shoreline of the Gulf of Mexico and the Caribbean (Taylor, 2012). These fish lay large, 2 mm diameter eggs (Huber, 2015) and are easy to care for in captivity where they will breed freely. The eggs are clear and the developing embryo can be easily observed by aid of a dissecting microscope, which make this fish an interesting and useful educational tool for use in both anatomy and embryology courses.

In spite of its self-fertilizing biology and relatively low genetic diversity (Tatarenkov et al., 2015), *K. marmoratus* remains phenotypically plastic. It survives in a wide range of water conditions, from soft acidic water to full strength sea water and can tolerate temperatures from 18 °C to 38 °C (Huber, 2015). It can survive long periods (days) immersed so long as it remains moist (Wright, 2012), and is often encountered in large numbers hiding above the water level, beneath bark and under logs in mangrove swamps (Taylor, 2012). This hardiness facilitates uncomplicated captive maintenance. *K. marmotatus* reaches a maximum size of 75 mm (Huber, 2015) and requires little space per individual fish meaning large colonies can be maintained without the need for large aquaria. More interesting still is that these fish, having lived as self-fertilizing hermaphrodites for much of their lives, can change into fully functional males that are able to spawn with and fertilize the eggs of hermaphrodite fish. Environmental conditions (such as temperature stress) can stimulate the development of males from eggs (Turner et al., 2006).

As the large eggs develop and without killing the organism, students can use a dissecting microscope to observe gastrulation, the development of the neural tube, somites and the circulation of blood. The embryos twitch when exposed to light, which means they can be used to facilitate a discussion of motor system synapse formation.

A detailed study of *K. marmoratus* embryology has been published by Mourabit

et al. (2011) as well as a means to manipulate the embryos for microscopic studies (Mourabit & Kudoh, 2012). The current article discusses the basic care of *K. marmoratus* in the teaching lab and presents micrographs of observations made using a dissecting microscope.

METHODS

Acquisition of fish

Fish were obtained from hobbyists within the American Killifish Association (http://www.aka.org) but they are also available from research labs such as my own or others such as Dr. Ryan Early (University of Alabama, http://rlearley.people.ua.edu/).

Fish Maintenance

Fish used in these studies were maintained in accordance with the ethics standards of Northwestern College, Iowa, and the University of Cape Town, South Africa.

Fish are housed individually in 500 mL plastic snap-fast tubs half-filled with a 14 g/L salt solution. The tubs are floated in an aquarium maintained at 25 °C (Fig. 1 A) with a 12 hour per day light cycle. A small hole punched into the lid of each tub is used for ventilation and to feed the fish. The fish are fed a staple diet of *Artemia nauplii*. The water in the tub should be refreshed at least once a week. Salt insensitive aquarium plants, such as Java moss and Java fern, were grown with the fish to aid in maintaining water quality.

Collecting eggs

The fish spawn daily and will deposit their eggs on fish spawning mops made from acrylic yarn (Fig. 1 B and C). Spawns number from 1 to 5 eggs at a time. This species is reported to eat its own eggs so the mops need to be checked several times a day and the eggs removed for incubation. The eggs are carefully handled by hand and placed on damp cotton wool to incubate (Fig. 1D) or into a petri dish with 14 g/L salt solution. Infertile eggs will turn a murky white and should be removed when they became visible. The eggs develop at room temperature (20–22 °C). To ensure a steady supply of eggs and to increase the probability of observing a specific developmental stage, several tubs of fish should be set up. Individual fish will take a break from spawning and these breaks are of unpredictable timing and duration.

Depending on incubation conditions and the strain used the fry hatched will hatch out between 20 and 40 days after the eggs are laid. The fry are large enough to eat *Artemia nauplii* on hatching and growth was rapid. The fry can be communally raised and then separated into individual tubs when adult. Fish raised together learn to tolerate each other but egg production is poor under such conditions. Once individual fish are separated into tubs of their own their territorial instincts dominate and the fish will not coexist with each other in the aquarium again.

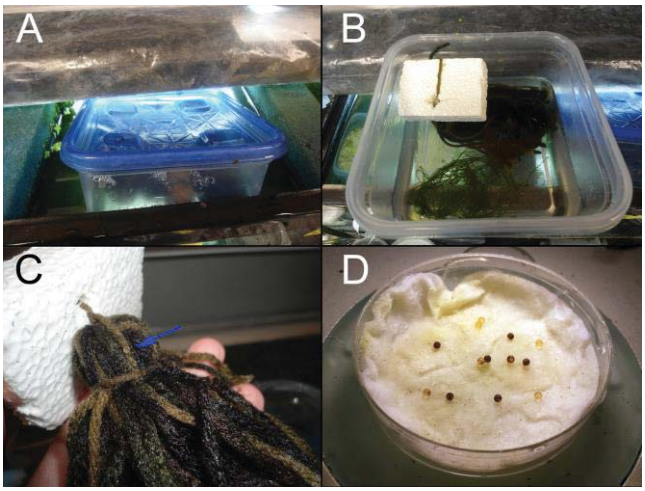

Figure 1: Captive spawning and maintenance of *Kryptolebias marmoratus*. A: Plastic tubs holding individual fish. The tubs are floated in a larger aquarium maintained at 25 ∘ C and 14 g/L salinity. B: Fish and spawning mop in a holding tub. C: Freshly laid egg in spawning mop. D: Eggs in various stages of development incubating on damp circular cotton wool pads. Embryos are visible in the eggs.

Microscopy

A Zeiss Primo Star microscope with AxioCam ICc 1 camera and AxioVision SE64 Rel V 4.9.10 software was used to capture images of the embryos. The 4× objective was used for most images to

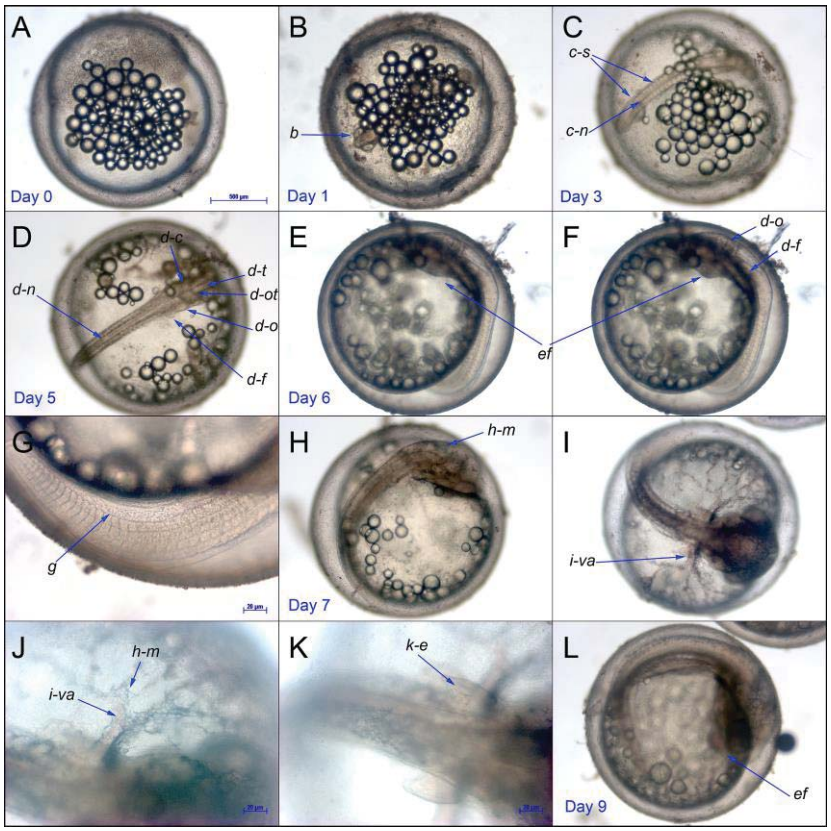

Figure 2: Developing embryos of *Kryptolebias marmoratus*. A: Day 0 embryo. Scale bar represents 500 µm. B: Day 1 embryo showing eye-buds (b) and brain development. C: Day 3 embryo showing somites (c-s) and notochord (c-n). D: Day 5 embryo showing nervous system structures (notochord, d-n; cerebellum, d-c; telencephalon, d-t; optic tectum, d-ot), otic vesicles (d-o) and fin buds (d-f). E–G: beating heat on Day 6 (ef); and unpigmented blood in the aorta (g). H-I: Day 7 embryo showing melanophores (h-m); and pigmented blood flowing through the vitelline artery (i-va). J & K: 10× objective images of pigmented blood in the vitelline artery (i-va) as well as melanophores migrating along the vitelline arteries (h-m) and erythrophores (k-e) on the developing pectoral fin. Scale bar represents 20 µm. L: Day 9 embryo with pigmented blood filled beating heart readily visible (ef).

simulate what can be observed using a regular bench dissecting microscope. The 10× objective was used for more detailed images.

Images were manipulated using Adobe Photoshop CS2. Individual images were pasted into an array and each image was manipulated using the image adjustment levels tool to set the white point for each image. Scale bars for each objective were calibrated using a stage calibration slide and then inserted into the published figure using Photoshop.

Use in a classroom

Eggs of day zero to day 9 were set out in the classroom in a 5 cm petri dish with enough water to cover the embryo. Dishes with eggs were positioned under a microscope for the students to view using a 4× objective.

Before viewing the embryos the students were shown images (Figure 2) and a brief explanation was given as to what they could observe in the eggs. Students were instructed to turn the microscope lamp off after viewing to prevent overheating of the embryos. Human embryological models were set out for comparison as well as selected illustrations from textbooks including the student's required textbook. While asking students questions about what they had seen, the microscope was checked to make sure it was still positioned correctly or that the embryo hadn't moved.

RESULTS

The eggs of *K. marmoratus* develop internally for the first day (Koenig & Chasar, 1984) and are laid at the gastrula stage. The images shown in Fig. 2 are labeled based on the day of collection starting at Day 0. In Fig. 2A a Day 0 embryo is visible. A large cell mass (gastrula) is visible. Twenty-four hours later (Fig. 2B) the head has begun to form (arrow b) and eye-buds are visible along with the ventricles in the eyes. Brain development is also visible with the brain furrow clearly defined as a dark shadow between the eye-buds. Somites are obscured by the lipid droplets. On Day 3 (Fig. 2C) the somites (c-

s) and notochord (c-n) are easily observed. It is also possible to distinguish the fore-, mid- and hindbrains (not shown). By this age the heart is beating but is difficult to discern. There is no blood circulation.

By Day 5 it is possible to discern the optic tectum (d-ot, Fig. 2D) as well as the cerebellum (d-c) and telencephalon (d-t). The otic vesicles (d-o) and fin buds of the developing pectoral fins (d-f) are also observable. The lipid droplets are now more diffuse and do not obscure the embryo as much as before. By Day 6 the beating heart is easily observed by orientating the embryos in profile (ef, Fig. 2E–F) and the blood, still unpigmented, can be seen to flow in the aorta of the embryo when viewed under higher magnification (10× objective, Fig. 2G).

By Day 7 (Fig. 2H–K) melanophores can be observed along the head (H, h-m) as well as the vitelline blood vessels extending into the yolk sac (J, h-m) as well as erythrophores in the developing fins (K, k-e) and along the dorsal surface. The erythrocytes in the blood vessels (I, i-va) are now pigmented. At this stage of development, and later, the blood vessels and erythrocytes are most easily observed in the vitelline artery (J, i-va). Erythrophores are visible in the fin buds of the embryos (K, f-e). The erythrophores and melanophores have a similar dendritic morphology with the latter having much longer dendrites. By Day 9 the heart is red with pigmented erythrocytes (Fig. 2L, ef) and can be easily observed to beat when the embryo is observed in profile.

As the embryo develops the pigmentation increases and features such as those described above become harder to observe. Late in development, the beating heart and blood coursing through the vitelline arteries are still easily observed. The eye (iris, pupil, sclera) are observable in mature embryos.

From Day 3 onwards the tail of the fish can be seen to twitch. On Day 5 the embryo is responsive to light. When illuminated under the microscope the heart rate will increase and the tail will twitch more regularly.

DISCUSSION

Eggs of *K. marmoratus* are large and easily observable using a dissecting microscope. The embryological features that are visible at 4× magnification are useful for the discussion and learning of important topics of embryological development such as blood circulation, brain development and body segmentation. The embryo is easily contrasted with human models to show evolutionary and functional similarities. The use of descriptive lecture, plastic models and the live embryos help the students to properly contextualize the sequence of embryonic development as well as the anatomical structures.

The idea for using *K. marmoratus* embryos came about as a means of avoiding the emotional disturbance experienced by medical students at observing chick embryos. Some students were upset by the idea that the chick was killed simply for observational purposes. Using the fish embryos avoids this ethical issue and needless killing of an organism. The embryos can be viewed without harm, and the eggs can go on to hatch.

The author has employed these embryos in classes for college students studying medicine (at the University of Cape Town) as well as biology, nursing and kinesiology anatomy (at Northwestern College, Iowa). The embryos have also been used in presentations for junior school children. Both college and junior school learners were excited to see the embryo twitch and its heartbeat.

The similarity between the fish embryo and early human embryos was apparent to the students. For many it was the first time they had seen a beating heart and they were awed to see the blood circulating. Amazement was expressed that the tiny embryo already had all the adult structures and that it would grow up to be a 75 mm fish. The speed of development also

impressed students. Tail flicks were observed by some students and was met with a sense of wonder. Many students were unable to observe the fin buds suggesting that instructors should be more explicit in pointing out the smaller structures in the pre-lab talk.

The demonstration of blood supply and the muscle twitching have served as useful starting points for the discussion of fetal development of the circulatory and nervous systems. The somites serve as a starting point for discussing segmentation in vertebrates, blood vessels and nerve development and how this relates to the scleratome, myotome and dermatome.

The presence of multiple chromophores in this species of fish can facilitate a discussion in human pigmentation and the use of different pigments by different species. While it isn't obvious by observing the embryo a discussion can still be held as to the origin of melanophores and melanocytes from the neural tube and the similarity between melanocytes and nervous tissues. The migration of the melanophores of the fish along the blood vessels also serves as a starting point for discussion of the arteries as highways for the migration of various cell types and structures, such as nerves.

There is great potential for the use of these fish in behavioral, ecological and environmental experiments. Readers are encouraged to read the review by Tayler (2012). For instance, it has been observed that the more polluted the water the more time the fish will spend out of the water (Bruce Turner, Dept. of Biology, Virginia Tech, pers comm). It would not be difficult performing such an experiment in a classroom setting.

The fish are able to live in very small volumes of water. Prof Bruce Turner maintains his experimental fish in stackable finger bowls (approximately 300 mL when filled). This means that it is possible to house many fishes for classroom experiments in a small space. The small water volume and living space do not constitute cruelty to this fish species, where in the wild they will often inhabit the burrows of crabs (Taylor, 2012). They can live for years in such small quarters without any sign of discomfort.

These fish will take most foods offered but because of the small volumes of water foods that might foul the water should be avoided. For this reason *Artemia nauplii* are used as a staple food. The fish are naturally amphibious (Pronko et al, 2013) and adventurous and without a secure lid on the tubs the fish will exit the tubs and aquarium to perish on the laboratory floor.

This small fish, with its ease of maintenance, is amenable to experimentation in a classroom setting and its large embryos are excellent vehicles for embryology teaching.

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REFERENCES

COOPER, C.A., WILSON, J.M., AND P.A. WRIGHT. 2013. Marine, freshwater and aerially acclimated mangrove rivulus (*Kryptolebias marmoratus*) use different strategies for cutaneous ammonia excretion. *Am J Physiol Regul Integr Comp Physiol* 304: 599–612.

HUBER, J.H. (editor). 2015. Killi-Data, chapter *Kryptolebias marmoratus*. Killi-Data International. URL: http://www.killi-data.org/mem12bers/marmoratRivu.php [Online; accessed 7-July-2015].

KOENIG, C.C., AND M.P. CHASAR. 1984. Usefulness of the hermaphroditic marine fish, *Rivulus marmoratus*, in carcinogenicity testing. *Natl Cancer Inst Monogr* 65: 15–33.

MOURABIT, S., EDENBROW, M., CROFT, D.P., AND T. KUDOH. 2011. Embryonic development of the self-fertilizing mangrove killifish *Kryptolebias marmoratus*. *Dev Dyn* 240: 1694–1704.

MOURABIT, S., AND T. KUDOH. 2012. Manipulation and imaging of *Kryptolebias marmoratus* embryos. *Integr Comp Biol* 52: 761–768.

POEY, F. 1880. Revisio piscium cubensium. *Anal Soc Espanola Hist Nat* 9: 243–261.

PRONKO, A.J., PERLMAN, B.M., AND M.A. ASHLEY-ROSS. 2013. Launches, squiggles and pounces, oh my! The water-land transition in mangrove rivulus (*Kryptolebias marmoratus*). *J Exp Biol* 216: 3988–3995.

TATARENKOV, A., EARLEY, R.L., PERLMAN, B.M., TAYLOR, D.S., TURNER, B.J., AND J.C. AYISE. 2015. Genetic Subdivision and Variation in Selfing Rates Among Central American Populations of the Mangrove Rivulus, *Kryptolebias marmoratus*. *J Hered* 106: 276–284.

TAYLOR, D.S. 2012. Twenty-four years in the mud: what have we learned about the natural history and ecology of the mangrove rivulus, *Kryptolebias marmoratus*? *Integr Comp Biol* 52: 724–736.

TURNER, B.J., FISHER, M.T., TAYLOR, D.S., DAVIS, W.P., AND B.L. JARRETT 2006. Evolution of 'maleness' and outcrossing in a population of the self-fertilizing killifish, *Kryptolebias marmoratus*. *Evol. Ecol. Res.* 8: 1475–1486.

WRIGHT, P.A. 2012. Environmental physiology of the mangrove rivulus, *Kryptolebias marmoratus*, a cutaneously breathing fish that survives for weeks out of water. *Integr Comp Biol* 52:7 92–800.